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13. ABSTRACT (Maximum 200 Words) The abnormal expression of Breast Cancer Specific Gene 1 (BCSG1) in malignant mammary epithelial cells is strongly linked to the development and progression of breast cancer. Our previous studies have identified DNA demethylation as an important mechanism for breast cancer cells to express BCSG1. We have demonstrated that similar to other protooncogenes such as c-myc, BCSG1 gene becomes selectively hypomethylated, which allows its abnormal expression in breast tumor cells. In turn, tumor cells expressing BCSG1 gain growth advantage. However, little is known about how BCSG1 exerts its oncogenic functions in breast cancer cells. In order to uncover the mechanisms whereby BCSG1 induces disease progression and malignant conversion, we have searched for BCSG1-interacting proteins through a yeast two-hybrid screening and co-immunoprecipitation assays. These studies led to the identification of BubR1, a critical mitotic checkpoint kinase, being the cellular target of BCSG1. We have also obtained new evidence from several lines of investigations to demonstrate that BCSG1 expression in breast cancer cells directly inhibits the mitotic checkpoint function and induces aneuploidy in a BubR1-dependent manner. These new findings suggest that induction of aneuploidy by BCSG1 is likely an important contributing factor to the development of advance-staged breast carcinoma.				
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Table of Contents

Cover.....	
SF 298.....	
Table of Contents.....	1
Introduction.....	2
Body.....	3
Key Research Accomplishments.....	5
Reportable Outcomes.....	5
Conclusions.....	
References.....	6
Appendices.....	8

Introduction

BCSG1 was originally identified through differential cDNA sequencing and was isolated from a human breast tumor cDNA library (1). BCSG1 gene maps to 10q23, is composed of five coding exons, and is transcribed into a mRNA of ~1 kb that encodes a 127-amino acid (AA) polypeptide (2). Comparison of the predicted protein sequence with genetic databases reveals that BCSG1 is highly homologous to a family of neuronal cytosolic proteins, namely synucleins that are mainly expressed in brain and are localized to presynaptic terminals (3-5)). BCSG1 shares 55.9% and 54.3% similarity, respectively, with synuclein α and synuclein β . The most conserved regions are in the N-terminal portion of the protein, consisting 6 repeated homologous domains of a KTKEGV consensus sequence. However, the C-terminal region (AA 86-127) of BCSG1 is quite different from synuclein α and β .

Being identified as a breast cancer specific gene, BCSG1 mRNA was detected in neoplastic breast epithelial cells but not in normal mammary epithelial cells (1). *In situ* hybridization analysis demonstrated a stage-specific expression pattern of BCSG1 mRNA varying from virtually no detectable expression in normal or benign breast tissues to low level and partial expression in low grade ductal carcinoma *in situ* (DCIS) to high expression in advanced infiltrating carcinomas. Recently, the stage-specific expression of BCSG1 mRNA is independently confirmed by RT-PCR analyses of tumor samples, showing that 79% of stage III/IV breast carcinomas were BCSG1-positive, whereas only 15% of stage I/II carcinomas expressed BCSG1 mRNA. Importantly, BCSG1 mRNA was undetectable in all benign breast lesions (6). Immunohistochemical studies to examine BCSG1 protein expression showed a similar pattern in that it was not detected in normal breast tissues, but was detected in a high percentage (70%) of stage III/IV breast ductal carcinomas (7). Analysis of breast tumor samples did not identify any sequence variation of BCSG1 gene from its original normal neuronal environment and no gene amplification was detected either (8). These studies suggested that transcriptional activation might account for its abundant expression in breast cancer cells. By cloning and analyzing the BCSG1 gene promoter region, we demonstrated that the DNA demethylation plays a major role for the active transcription of BCSG1 in breast cancer cells (9; 10). The exon 1 of BCSG1 gene contains a CpG island that is tensely methylated in normal breast tissues and in breast cancer cell lines that do not express this protein. However, in BCSG1-positive breast tumors and tumor-derived cell lines, the CpG island is totally demethylated that allows RNA polymerase and other transcription factors to access the promoter and to initiate the gene transcription. At present, it is not fully clear what genetic and cellular factors control the methylation status of this oncogene. However, the fact that BCSG1 gene becomes selectively demethylated, which allows its abnormal expression in advanced-staged breast tumors implies that this oncogene product may actively participate in the process of disease progression. This speculation is strongly supported by a study showing that overexpression of BCSG1 in breast cancer cell line MDA-MB435 cells led to a significant increase in motility and invasiveness in cell culture and a profound augmentation of metastasis in nude mice (11). In addition to stimulating breast cancer cell invasion and metastasis, BCSG1 is also positively involved in cell proliferation. We showed that exogenous expression of BCSG1 in MCF-7 and MDA-MB435 cells significantly stimulated the cell growth under anchorage-dependent and independent conditions (12). Conversely, blocking the endogenous BCSG1 expression in T47D cells by BCSG1 antisense inhibited the colony formation of T47D cells in soft agar (13).

Since during the first year of grant support we have clearly elucidated the major molecular mechanisms that control the abnormal expression of BCSG1 in breast cancer cells, we decided to conduct some investigations to get some clues about how BCSG1 expression induces breast cancer disease progression and malignant transformation. In this report, we show that the oncogenic protein BCSG1 directly interacts with BubR1, an important mitotic checkpoint kinase. We demonstrate that BCSG1 expression directly inhibits mitotic checkpoint function and induces aneuploidy in BCSG1-stably expressing cells. These new findings are now published in the journal of oncogene.

BODY

To elucidate the cellular mechanisms underlying the effects of BCSG1 in breast cancer cells we used a yeast two-hybrid system to screen for proteins that could associate with BCSG1. Through this screening, we identified the mitotic checkpoint protein BubR1 as a novel binding partner of BCSG1. The specific association of BCSG1 with BubR1 in breast cancer cells was demonstrated by immunoprecipitation and GST pulldown assays. Intriguingly, experiments conducted in 3 different cell lines all showed that exogenous expressions of BCSG1 consistently reduce the cellular levels of the BubR1 protein without affecting BubR1 mRNA expression. The tendency of endogenous BCSG1 expression coinciding with lower BubR1 protein levels was also observed in 7 out of 8 breast cancer cell lines. We further showed that the reducing effect of BCSG1 on BubR1 protein expression could be prevented by treating BCSG1-transfected cells with MG132, a selective 26S proteasome inhibitor, implying that the proteasome machinery may be involved in the BCSG1-induced reduction of the BubR1 protein. Accompanied with a reduction of BubR1 protein level, BCSG1 expression resulted in multinucleation of breast cancer cells upon treatment with spindle inhibitor nocodazole, indicating an impaired spindle assembly checkpoint. Taken together, our novel findings suggest that BCSG1 may promote the progression of breast cancer at least in part by compromising the mitotic checkpoint control through inactivation of BubR1.

For detailed results and figures, please see attached reprint.

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of BCSG1 stable breast cancer cell lines (MCF7-BCSG1 vs. MCF7-neo; MDAMB435-BCSG1 vs. MDAMB435-neo).

REPORTABLE OUTCOMES

- One manuscript has been published in the second year of the award.

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Breast cancer-specific gene 1 interacts with the mitotic checkpoint kinase BubR1

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The abnormal expression of breast cancer-specific gene 1 (BCSG1) in malignant mammary epithelial cells is highly associated with the development and progression of breast cancer. A series of *in vitro* and *in vivo* studies performed in our laboratory and others have demonstrated that BCSG1 expression significantly stimulates proliferation, invasion, and metastasis of breast cancer cells. However, currently little is known about how BCSG1 exerts its oncogenic functions. To elucidate the cellular mechanisms underlying the effects of BCSG1 in breast cancer cells, we used a yeast two-hybrid system to screen for proteins that could associate with BCSG1. Through this screening, we identified the mitotic checkpoint protein BubR1 as a novel binding partner of BCSG1. The specific association of BCSG1 with BubR1 in breast cancer cells was demonstrated by immunoprecipitation and GST pull-down assays. Intriguingly, experiments conducted in four different cell lines all showed that exogenous expressions of BCSG1 consistently reduce the cellular levels of the BubR1 protein without affecting BubR1 mRNA expression. The tendency of endogenous BCSG1 expression coinciding with lower BubR1 protein levels was also observed in seven out of eight breast cancer cell lines. We further showed that the reducing effect of BCSG1 on BubR1 protein expression could be prevented by treating BCSG1-transfected cells with MG-132, a selective 26S proteasome inhibitor, implying that the proteasome machinery may be involved in the BCSG1-induced reduction of the BubR1 protein. Accompanied with a reduction of BubR1 protein level, BCSG1 expression resulted in multinucleation of breast cancer cells upon treatment with spindle inhibitor nocodazole, indicating an impaired mitotic checkpoint. Taken together, our novel findings suggest that BCSG1 may accelerate the progression of breast cancer at least in part by compromising the mitotic checkpoint control through inactivation of BubR1. *Oncogene* (2003) 22, 7593–7599. doi:10.1038/sj.onc.1206880

Keywords: BCSG1; BubR1; mitotic checkpoint; yeast two-hybrid; aneuploidy; multinucleation

Introduction

Previously, by differential DNA sequencing and *in situ* hybridization, BCSG1 has been identified as a breast cancer-specific gene. BCSG1 is not expressed in normal breast tissue or tissues with benign breast diseases, but it is highly expressed in the vast majority of the advanced staged breast carcinoma (Ji *et al.*, 1997; Bruening *et al.*, 2000). Several lines of evidence suggest that BCSG1 plays a positive role in the process of invasion and metastasis of breast cancer cells. *In vitro* studies demonstrate that ectopic expression of BCSG1 in breast cancer cells significantly stimulates cell proliferation and cell migration (Jia *et al.*, 1999; Liu *et al.*, 2000), whereas blocking endogenous BCSG1 expression markedly reduces the anchorage-independent growth of breast cancer cells (Lu *et al.*, 2002). In nude mice, overexpression of BCSG1 resulted in a profound augmentation of metastasis (Jia *et al.*, 1999). A recent study further revealed additional oncogenic functions of BCSG1 that promotes tumor cell survival under adverse conditions and increase cancer cell resistance to certain chemotherapeutic drugs (Pan *et al.*, 2002). Despite the mounting evidence that links BCSG1 with breast tumorigenesis, currently little is known about how BCSG1 exerts these oncogenic functions. Recently, an immunostaining study conducted by Surguchov *et al.*, 2001 has shown that BCSG1 is localized to poles of spindle in mitotic cells and suggested that BCSG1 might be a centrosomal protein.

The mitotic checkpoint monitors the proper assembly of the mitotic spindle through a set of proteins that includes Mad1, Mad2, Bub1, Bub3, and BubR1 (Chan *et al.*, 1998; Jablonski *et al.*, 1998; Sudakin *et al.*, 2001; Canman *et al.*, 2002; Shannon *et al.*, 2002). Damage to mitotic spindle fibers can activate these mitotic checkpoint genes, whose products arrest cells in mitosis. Mutations in any of these genes result in failure to arrest the cell cycle at G2-M, and cells exit mitosis prematurely (Roberts *et al.*, 1994; Paulovich *et al.*, 1997; Hoyt, 2001). Thus, the loss of the mitotic checkpoint machinery may cause aneuploidy that is frequently observed in many types of human cancer. Genetic disruption of this checkpoint in mouse embryos causes lethality due to missegregation of chromosome (Dobles *et al.*, 2000). Studies reported by Taylor and Mckee (1997) has shown that overexpression of a dominant-negative Bub1

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mutant resulted in over-riding the mitotic checkpoint control and cells continued to progress through cell cycle leading to multinucleation. Human BubR1 and Bub1 are structurally related and both proteins possess a kinase domain in the C-terminus (Taylor and McKeon, 1997; Chan *et al.*, 1999; Taylor *et al.*, 2001). BubR1 is ubiquitously expressed throughout the cell cycle and undergoes phosphorylation during mitosis (Chan *et al.*, 1999). After the cells exit from mitosis, BubR1 levels decrease (Davenport *et al.*, 1999). The key target of the mitotic checkpoint is the anaphase-promoting complex (APC), a ubiquitin ligase (Page and Hieter, 1999). The activation of APC ubiquitin ligase activity by Cdc20 is required for APC to control sister chromatid separation and exit from mitosis (Sudakin *et al.*, 2001; Tang *et al.*, 2001). BubR1 has been shown to inhibit APC through direct binding to Cdc20 (Skoufias *et al.*, 2001; Tang *et al.*, 2001; Chen, 2002; Fang, 2002). The essential role of BubR1 in mitotic checkpoint was demonstrated by the study showing that inactivation of BubR1 by microinjection of specific antibodies abolished the checkpoint control (Chan *et al.*, 1998, 1999).

Despite the importance of BubR1 in mitotic checkpoint, BubR1 mutation is very rare in human cancer including breast cancer (Myrie *et al.*, 2000; Fagin, 2002; Ouyang *et al.*, 2002; Shichiri *et al.*, 2002). A recent study has found a correlation of decreased mRNA expressions of BubR1 and Bub1 with lymph node metastasis of colon carcinomas, suggesting the existence of nonmutational inactivation mechanisms for BubR1 (Shichiri *et al.*, 2002). In the present study, we show that the oncogenic protein BCSG1 directly interacts with BubR1. This association appears to degrade BubR1. Consequently, the cells expressing BCSG1 display signs of aneuploidy such as multinucleation and micronucleation upon treatment with microtubule-destabilizing drug nocodazole.

Results and discussion

By conducting a yeast two-hybrid screening to identify BCSG1 interacting proteins, we isolated a positive clone that showed interactions with the full-length (amino acids (aas) 1–127) as well as the C-terminal domain of BCSG1 (aas 86–127). Sequencing analysis determined that this cDNA clone contains a 1.4 kb cDNA of the human BubR1 starting at the aa 606 in frame with the Lex A coding sequence. Since BubR1 is a critical component of the mitotic checkpoint control, it could be a potential cellular target of oncogenic proteins such as BCSG1 that induces tumor progression potentially through mechanisms of disturbing genome stability. To confirm BCSG1 and BubR1 interaction in mammalian cells, initially we performed immunoprecipitation (IP) assay using total cell lysates isolated from a BCSG1-positive cell line T47D and a BCSG1-negative cell line HepG2 (Lu *et al.*, 2001). Using BCSG1-specific antibody to IP, BubR1 was only detected in the IP complex of T47D cells (Figure 1a, lane 2) but not in the

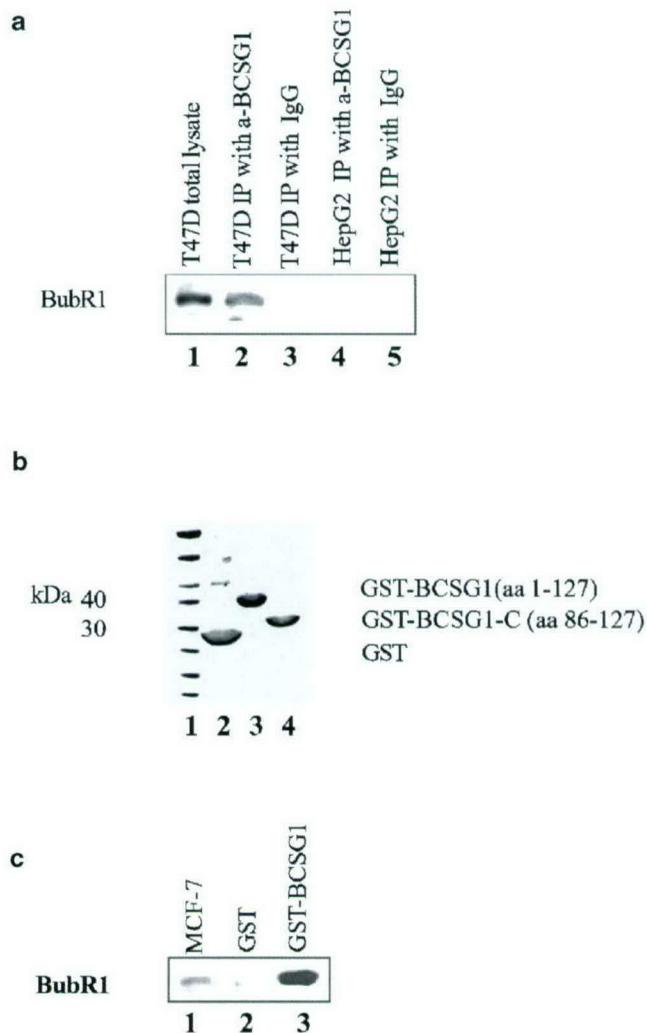


Figure 1 Interaction of BCSG1 with human BubR1. (a) Immunoprecipitation with anti-BCSG1 antibody: T47D and HepG2 cell lysates were immunoprecipitated with either IgG (lanes 3, 5) or a goat anti-BCSG1 antibody (lanes 2, 4). After extensive washings, immunoprecipitates were separated by SDS-PAGE and transferred to a nitrocellulose membrane and probed with a rabbit anti-BubR1 antibody. T47D total cell lysate (lane 1) was used as a positive control for BubR1 detection. (b) Expression of GST-BCSG1 fusion proteins: 5 μ g of purified GST fusion proteins isolated from bacterial cell lysates were analysed with Western blot using anti-GST antibody. The membrane was subsequently probed with anti-BCSG1 antibody that confirmed the BCSG1 expression (data not shown). (c) GST-pull down assay: GST alone (lane 2) or GST-BCSG1 fusion protein (lane 3) was incubated with glutathione agarose for 1 h. After three washes with PBS, the immobilized GST or GST-BCSG1 fusion proteins was incubated with 1 mg of MCF-7 cell lysate for 2 h. The beads were extensively washed and boiled in sample buffer. The pelleted proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with a rabbit anti-BubR1 antibody. MCF-7 total cell lysate (lane 1) was used as a positive control for BUBR1 detection

IP complex formed with the HepG2 cell lysate (Figure 1a, lane 4), despite the detection of BubR1 protein in the total cell lysate of HepG2 (data not shown). BubR1 was also not detected in the IP complex

with the control IgG (Figure 1a, lanes 3 and 5). We had some difficulties to clearly detect BCSG1 in the BubR1 IP complex by Western blotting because of the overlapping signals of BCSG1 and the IgG light chain. Thus, in order to further confirm BCSG1 and BubR1 interaction, we generated and purified glutathione-S-transferase (GST) fusion proteins that contain the full-length (aas 1–127) or the C-terminal domain of BCSG1 (aas 86–127) (Figure 1b). GST pull-down assay was performed with purified GST-fusion proteins immobilized on glutathione-agarose beads and MCF-7 cell lysate. After intensive washings, proteins bound to the beads were eluted and analysed by Western blotting with a rabbit anti-BubR1 antibody. Figure 1c shows that BubR1 did not bind to GST (lane 2). However, a strong band of BubR1 was detected with GST-BCSG1 (lane 3). The pull-down assay also demonstrated an interaction of BubR1 with the GST fusion protein containing the C-terminal domain of BCSG1 (data not shown). Taken together, these results confirmed the finding of the yeast two-hybrid and demonstrated the specific interaction of BCSG1 with BubR1 in breast cancer cells.

In an attempt to explore the functional consequences of this interaction, BCSG1-negative MCF-7 cells were transfected with pCI-BCSG1, or the empty vector pCIneo and the pooled transfectants were selected by neomycin resistance to generate stable cell lines. The total cell lysates of these two cell lines were analysed for BCSG1 and BubR1 expression by Western blotting. The left panel of Figure 2a shows that the level of BubR1 protein of MCF7-BCSG1 cells is significantly lower (by 70%) than that of MCF7-neo cells. To determine that this observation is not only confined to MCF-7 cells, pCI-BCSG1 and the control vector pCIneo were transiently transfected into the BCSG1-negative MDA-MB435 cells. At 2 days after transfection, cells were harvested for Western blotting to detect BCSG1 and BubR1 expression. The right panel of Figure 2a shows that BCSG1 expression led to a twofold reduction of BubR1 protein level in MDA-MB435 cells. The moderate reduction of BubR1 in MDA-MB435 cells could be explained by the fact that only 20–30% of cells were transfected. The transfection efficiency was determined by transfection of a plasmid expressing the green fluorescent protein (GFP) in a parallel experiment. The reducing effects of BCSG1 expression on cellular BubR1 protein levels were also evident in MCF10A and HeLa cells after transient transfection of pCI-BCSG1 plasmid DNA. Thus, these results obtained from four individual cell lines consistently demonstrate a reduction of BubR1 protein after BCSG1 expression.

To exclude the possibility that BCSG1 expression might somehow indirectly affect BubR1 mRNA transcription or stability, we performed RT-PCR analysis to compare the BubR1 mRNA levels in MCF7-BCSG1 and MCF7-neo cells. By utilizing two sets of specific primers corresponding to the 5' region (nt 755–1061) and the 3' region (nt 2874–3305) of the BubR1 mRNA sequence, we show that BubR1 mRNA levels were not reduced in cells expressing BCSG1 (Figure 2b). This

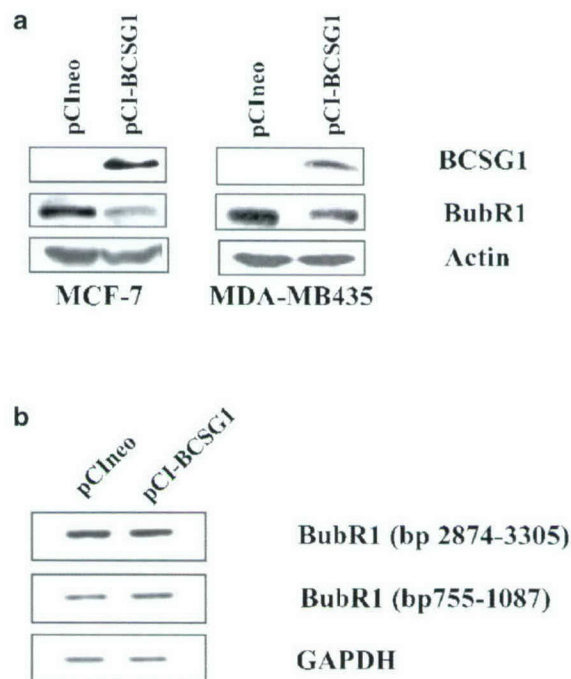


Figure 2 Reduction of BubR1 protein levels in cells expressing BCSG1. (a) Western blot analysis was conducted to examine BubR1 expression in pCI-BCSG1 and pCIneo stably transfected MCF-7 cells (left panel) and in MDA-MB435 (right panel) cells that were transiently transfected with these two vectors. After probing with anti-BubR1, the same blot was stripped and reprobed with anti-BCSG1 antibody and subsequently with anti-actin antibody as a control. The graphs shown are representative of 3–5 independent experiments. (b) BubR1 mRNA expressions in MCF-7-neo and MCF-7-BCSG1 were examined by RT-PCR analysis using BubR1-specific primers from two different regions. Total RNA (1 μ g) was used in the reaction of RT in a volume of 20 μ l. The RT product (2 μ l) was used in PCR with specific primers to BubR1 or GAPDH. The RT-PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. The RT-PCR assays with fewer cycles of amplification also did not detect differences regarding the levels of BubR1 mRNA expression in BCSG1 and neo clones

result indicates that the decrease of BubR1 protein in cells expressing BCSG1 does not result from a lower mRNA expression.

To ensure that the observed inverse correlation of expression between BCSG1 and BubR1 in transfected cells is not an artefact of transfection, we examined the endogenous expression of both proteins in eight breast cancer cell lines by Western blotting. As illustrated in Figure 3, three BCSG1-negative cell lines including H3396 (lane 3), MCF7 (lane 5), and MDA-MB435 (lane 7) have higher levels of BubR1 (2–3-fold) as compared to four BCSG1-positive cell lines including AU565 (lane 1), H3922 (lane 4), MDA-MB 231 (lane 6), and T47D (lane 8). In H3922 cells, the anti-BubR1 antibody only detected a band of slower mobility that may represent the phosphorylated BubR1. The expression of BubR1 in BT-20 cells does not appear downregulated by BCSG1 expression. Thus, seven out of eight breast cancer cell lines (87.5%) exhibit an inverse relationship between

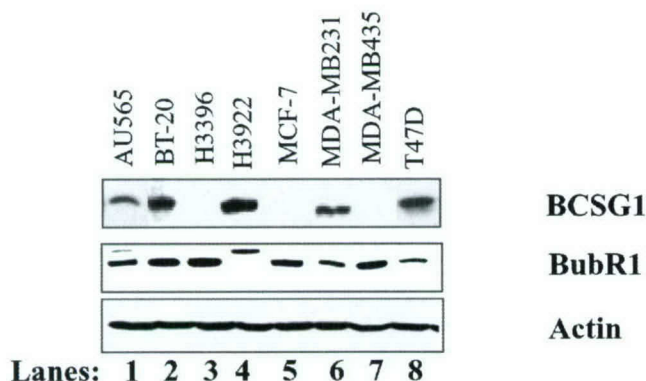


Figure 3 Inverse correlation of BCSG1 expression with lower BubR1 protein levels in different breast cancer cell lines. Western blot analysis was conducted to examine the BubR1 and BCSG1 expressions in eight different breast cancer cell lines. Total cell lysate (20 μ g) from each cell line was used

endogenous BCSG1 expression and the amount of BubR1 protein, thereby complementing the findings obtained by ectopic expressions of BCSG1.

Based on the above results, we speculate that the direct interaction of BCSG1 with BubR1 may somehow lead to the degradation of BubR1 protein. To this end, we used the compound MG-132, a specific inhibitor of the 26S proteasome, in our study to determine whether this inhibitor could block the BCSG1-mediated reduction of BubR1. MG-132 has been used in other studies that demonstrate the involvement of the ubiquitin-proteasome pathway in some components of the mitotic checkpoint (Page and Hieter, 1999). A myc-tagged BubR1 expression vector (pCS2-BubR1) was cotransfected with pCI-BCSG1 or with the control vector pCI-neo into MCF10A or MCF-7 cells that do not express endogenous BCSG1. At 2 days after transfection, cells were trypsinized and were reseeded in two 100 mm dishes at an equal density. Cells were allowed to adhere for 16 h and one dish was treated with DMSO and the other one was treated with MG-132 for 6 h. Total cell lysates from transfected cells were then harvested and examined for the myc-BubR1 expression with anti-myc antibody. Figure 4 shows that compared with pCI-neo-transfected MCF-7 (lane 1) and MCF10A cells (lane 5) BCSG1-transfected cells in both cell lines had lesser amount of myc-BubR1 (lane 3 vs lane 1 and lane 7 vs lane 5). MG-132 treatment lowered the amount of myc-BubR1 protein in the mock-transfected cells (lanes 2 and 6). In contrast, MG-132 increased the levels of myc-BubR1 protein in cells expressing BCSG1 (lane 4 vs lane 3 and lane 8 vs lane 7).

BubR1 monitors the kinetochore attachment to the spindle. It has been shown that HeLa cells expressing the dominant-negative mutant of Bub1 continue to progress through cell cycle and become large and multinucleated (Taylor and McKeon, 1997). We were interested to know whether the downregulation of BubR1 results in an impairment of the spindle assembly checkpoint in cells overexpressing BCSG1. To investi-

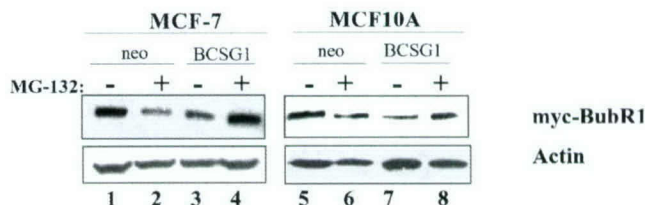


Figure 4 Reversal of BCSG1-induced degradation of BubR1 protein by MG-132. MCF-7 and MCF10A cells were transiently transfected pCS2-BubR1 (encoding a myc-tagged BubR1) with pCI-BCSG1 or with pCI-neo. At 2 days after transfection, transfected cells were trypsinized and were reseeded in two 100 mm dishes at an equal density. Cells were allowed to adhere for 16 h and one dish was treated with DMSO and the other one was treated with MG132 at 10 μ M concentration for 6 h for each cell line. Total cell lysates were then harvested and examined for the myc-BubR1 expression with anti-myc antibody. The data shown are representative of two separate experiments

gate this, we used microtubule-destablizing drug nocodazole that arrests the cells in mitosis by inhibiting the spindle dynamics. Parental MCF-7 cells, MCF7-BCSG1 and MCF7-neo clones were treated with 0.5 μ M of nocodazole. After 30 h of treatment, cells were examined microscopically and the mitotic and multinucleated cells were quantitated. Figure 5a shows that while the morphology of MCF-7 cells was not changed by BCSG1 expression under normal culture conditions, BCSG1 expression resulted in different responses to nocodazole. The percentage of mitotic arrested cells after nocodazole treatment was significantly lower in MCF7-BCSG1 as compared to parental MCF-7 and the neo cells. More strikingly, a large portion of MCF7-BCSG1 cells (34.3%) became large and multinucleated, whereas under same treatment only less than 10% neo and untransfected MCF-7 cells displayed multinucleation (Figure 5b).

To confirm these findings, nocodazole treatment was applied to BCSG1 and neo-transfected MDA-MB435 cells. Similar to MCF-7 cells, BCSG1 expression markedly increased multinucleation upon nocodazole treatment. A significantly different response was shown after 24 h treatment of nocodazole, and the difference in the percentages of multinucleated cells persist after 30 h treatment (Figure 5c). These observations suggest that expression of BCSG1 leads to over-riding the mitotic arrest caused by nocodazole and the cells continue to progress through cell cycle under nocodazole treatment resulting in multinucleation.

In order to directly demonstrate that the inhibited BubR1 function in the mitotic checkpoint control due to its interaction with BCSG1 contributes to the multinucleation, we overexpressed myc-BubR1 in MCF-7, MCF7-neo, and MCF7-BCSG1 cells by transient transfection of pCS2-BubR1 along with pEGFP as a marker of transfection. At 24 h after transfection, cells were treated with nocodazole for 30 h. The cells were fixed, stained with DAPI, and examined microscopically. Figure 6 shows that exogenous expression

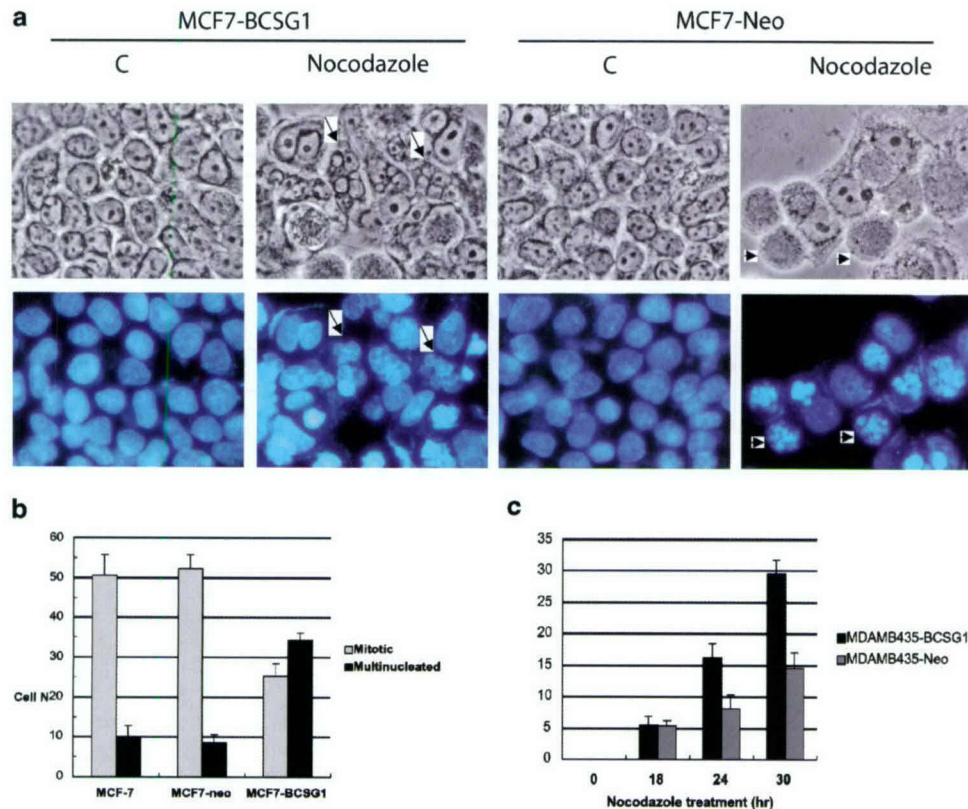


Figure 5 BCSG1 expression induces multinucleation in MCF-7 and MDA-MB435 cells upon nocodazole treatment. (a) MCF-7-neo and MCF-7-BCSG1 cells were treated with 0.5 μ M of nocodazole for 30 h. Cells were fixed with cold methanol, washed with PBS, and stained with DAPI. The photographs were taken using the Penguin 600CL digital camera at a magnification of 200. The arrows indicate the large and multinucleated cells and the arrowhead indicates mitotic cells. (b) Parental MCF-7, MCF-7-neo, and MCF-7-BCSG1 cells were treated with 0.5 μ M of nocodazole for 30 h. For each sample, 200–300 cells randomly chosen from five different views were scored for interphase, mitotic, or multinucleated. The induction of multinucleated cells by nocodazole in MCF7-BCSG1 cells was also dose- and time-dependent. (c) MDAMB435-BCSG1 and MDAMB435-neo stable clones were treated with 0.5 μ M of nocodazole for different times as indicated. For each sample, 400–500 cells randomly chosen from five different views were scored for mono nucleated or multinucleated

of myc-BubR1 did not alter the responses to the nocodazole-induced mitotic arrest in MCF-7 and neo clone. In contrast, myc-BubR1 expression in MCF7-BCSG1 cells significantly increased the mitotic index and drastically reduced the percentage of multinucleated cells to levels similar to MCF-7 and the neo clone. These data provide additional evidence that links the impaired mitotic checkpoint function to the decreased BubR1 protein level by BCSG1 expression.

Previous studies have shown that various types of cancers have defects in mitotic checkpoint, which in some cases could be caused by silencing of Bub1 and/or BubR1 genes due to mutations (Ohshima *et al.*, 2000). Results from our study for the first time suggest that BubR1 may be a cellular target of oncogenic proteins such as BCSG1 that induces BubR1 degradation as a new mechanism for inactivation of the mitotic checkpoint. BCSG1 expression has been strongly correlated with breast cancer disease progression. The ability of BCSG1 to interact with BubR1, an important component of the mitotic checkpoint, may provide a mechanism whereby overexpression of this protein in breast

cancer leads to genomic instability which is an important driving force in tumor progression.

Materials and methods

Yeast two-hybrid screening

A yeast two-hybrid system and a cDNA library of SKBR-3 were purchased from Origene Technologies (Rockville, MD, USA), and the yeast two-hybrid screening was performed as per the manufacturer's instructions. Briefly, BCSG1 coding region was amplified by PCR from the expression vector pCI-BCSG1 with the primer BCSG-Y1 5' CTCGAGGAATT-CATGGATGTCTTCAAGAA 3' (sense) and BCSG-Y3 5' ACCGGATCCCCTCTAGTCTCCCCCACTCTGGGCCTC 3' (antisense) and cloned into pEGY202 vector at *Eco*R1 and *Bam*H1 sites in correct reading frame to express Lex A-BCSG1 fusion protein. The resulting plasmid vector PEG202-BCSG1 was cotransformed with pSH18-34 lacZ reporter plasmid into yeast strain EGY194. After selection, one of these transformants was transformed with the SKBR-3 cDNA library plasmid DNA and screened for positive clones. Positive AD library plasmids thus obtained were reintroduced into the

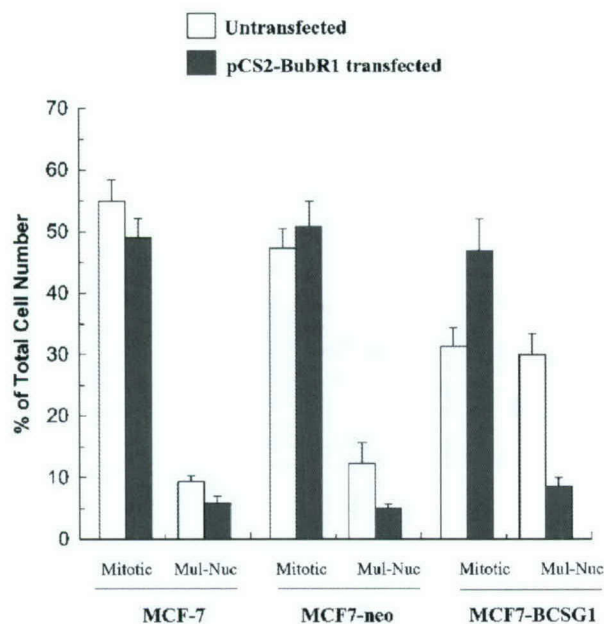


Figure 6 Overexpression of myc-BubR1 restored the mitotic checkpoint control in MCF7-BCSG1 cells. MCF-7, MCF7-neo, and MCF7-BCSG1 seeding in the culture glass chamber were untransfected or transfected with pCS2-BubR1 and pEGFP. At 24 h post-transfection, nocodazole at 0.5 μ M concentration was added for 30 h and cells were fixed by cold methanol. Equal transfection efficiency was indicated by similar numbers of green cells in different cell lines. Mitotic and multinucleated (Mul-Nuc) cells were scored as described in Figure 5

original yeast strain to conform the interaction. The screening of 1×10^6 independent clones identified 12 positive clones.

The cDNA insert of plasmid isolated from all positive clones was sequenced to identify the gene.

IP

Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% NP-40, and 0.5% sodium deoxycholate) in the presence of protease inhibitors. Cell lysate (1 ml) containing 500 μ g of protein was precleared with isotype-matching IgG for 1 h and then incubated with 2 μ g of anti-BCSG1 antibody (Santa Cruz, sc-10698) at 4°C for 2 h. A/G sepharose (30 μ l of protein) was then added to the lysate and the mixture was further incubated for 1 h. After washes, proteins bound to the beads were eluted with SDS sample buffer by boiling, separated by SDS-PAGE, and analysed by Western blotting with rabbit anti-BubR1 antibody.

Production and purification of GST-tagged BCSG1

The full-length BCSG1 (aas 1–127) and the C-terminal domain of BCSG1 (aas 86–127) were separately cloned into pEGX-4T.1 vector (Pharmacia) and transformed into BL-21 cells. Production of the fusion protein was induced in 500 ml culture by addition of isopropyl- β -D-thiogalactoside to a final

concentration of 1 mM. After a 2 h induction, the cells were harvested and lysed by sonication in lysis buffer (Wang *et al.*, 1991) and GST protein was purified by using glutathione-sepharose 4B beads. Purified proteins were stored at –80°C and 5 μ g per sample was analysed by Western blotting with anti-GST and anti-BCSG1 antibodies.

GST pull-down assay

GST-BCSG1 (30 μ g) protein or GST alone was added to 50% slurry of glutathione-sepharose 4B and incubated for 30 min. The unbound protein was then washed three times with PBS containing 1% Triton X-100. MCF-7 cell lysate (1 mg) was mixed with the beads and incubated for 1 h at 4°C. After extensive washes, the beads were collected, boiled in sample buffer. The proteins were analysed by Western blotting.

RT-PCR analysis of BubR1 mRNA

MCF-7-BCSG1 and MCF-7-neo stable transfected cells were established by transfection of pCIneo-BCSG1 or the control vector pCIneo and G418 selection (Gupta *et al.*, 2003). Total RNA was isolated from MCF-7-BCSG1 or MCF-7-neo cells using the Ultraspec RNA reagent (Biotecx Laboratories, Houston, TX, USA). The RT was conducted with random primers (Promega) using Superscript II (Invitrogen). A 333 bp DNA fragment corresponding to the region of 755–1087 of BubR1 cDNA sequence was obtained by PCR using the primer set Bub1b-2-F (5'CAAGAGCTCCAATCATCCG-TGTAGG 3') and Bub1b-2-R (5'GTGCAGTCTCTTCCACATATGGAGTG 3'). In addition, a 432 bp DNA fragment corresponding to the 3' region of the BubR1 cDNA sequence (2874–3305) was obtained by PCR using the primer set Bub1b-1-F (5'CTGTTCTTCTCCCTACCAGGTAG 3') and Bub1b-1-R (5'GGTAGTGCATCTAAATGTGTCCT 3'). The PCR reaction of 30 cycles was carried out at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s with a final extension at 72°C for 5 min. Detection of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA by RT-PCR from the same sample was performed as previously described (Lu *et al.*, 2002).

Abbreviations

aa, amino acid; APC, anaphase-promoting complex; BCSG1, breast cancer-specific gene 1; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione-S-transferase; IP, immunoprecipitation.

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